

# Evaluation of Systemic Thiol/Disulfide Homeostasis as a Novel Tool for the Assessment of Oxidative Stress in Patients with Retinopathy of Prematurity

## Prematüre Retinopatisi Hastalarında Oksidatif Stresin Değerlendirilmesinde Sistemik Tiyol/Disülfid Homeostazının Yeni Bir Araç Olarak İncelenmesi

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### ABSTRACT

**Purpose:** To investigate systemic oxidative stress by evaluating serum dynamic thiol/disulfide concentrations using a novel automated homeostasis assay in retinopathy of prematurity (ROP) patients.

**Materials and methods:** Patients (n = 71) with a birth weight less than 1,500 g or gestational age less than 32 weeks were recruited. Groups were categorized based on those who developed ROP (n=48) and those without ROP (controls) (n = 23). The native and total thiol, disulfide levels as well as disulfide-to-native, disulfide-to-total thiol, and native thiol-to-total thiol ratios were examined and analyzed between the ROP and control groups using a novel automated spectrophotometry assay.

**Results:** Levels of disulfide as well as the ratios of disulfide/native thiol and disulfide/total thiol were augmented, while the native/total thiols were reduced significantly in the ROP patients when compared to controls (mean  $\pm$  SD,  $19.6 \pm 7.4$  vs.  $14.4 \pm 3.4$   $\mu\text{mol/L}$ ,  $p < 0.001$  for disulfide, median (IQR),  $5.8$  ( $3.8-8.1$ ) vs.  $4.3$  ( $3.7-5.8$ )  $\mu\text{mol/L}$ ,  $p = 0.017$  for disulfide/native thiol, median (IQR),  $4.7$  ( $3.3-6.1$ ) vs.  $3.7$  ( $3.2-4.7$ )  $\mu\text{mol/L}$ ,  $p = 0.017$  for disulfide/total thiol, median (IQR),  $80.9$  ( $75.3-86.5$ ) vs.  $85.1$  ( $81.0-87.1$ )  $\mu\text{mol/L}$ ,  $p = 0.017$  for native thiol/total thiol levels). There were no significant differences between ROP stages and any of the thiol-disulfide parameters ( $p > 0.05$  for all values).

**Conclusion:** These findings showed that the thiol/disulfide homeostasis was altered in the ROP patients. This metabolic imbalance may be important in the ROP etiology.

**Key words:** Native thiol, oxidative stress, retinopathy of prematurity, thiol/disulfide homeostasis, total thiol.

### ÖZ

**Amaç:** Prematüre retinopatisi (ROP) hastalarında yeni bir otomatik homeostaz analizi kullanımı ile serum dinamik tiyol/disülfid konsantrasyonlarını değerlendirilerek sistemik oksidatif stresi araştırmak.

**Gereç ve yöntemler:** Doğum ağırlığı 1.500 gramdan düşük veya gebelik haftası 32 haftadan küçük olan hastalar (n = 71) toplandı. Gruplar, ROP gelişenlere (n = 48) ve ROP olmayanlara (kontroller) (n = 23) göre kategorize edildi. ROP ve kontrol grupları arasında yeni bir otomatikleştirilmiş spektrofotometrik analiz kullanılarak natif tiyol, total tiyol ve disülfid seviyeleri ile birlikte disülfid/natif tiyol, disülfid/total tiyol ve natif/total tiyol oranları incelendi ve analiz edildi.

**Bulgular:** ROP hastalarında, kontrol grubu ile karşılaştırıldığında disülfid seviyeleri ile birlikte disülfid/natif tiyol ve disülfid/total tiyol oranları istatistiksel olarak anlamlı oranda artmış olup natif/total tiyol oranları anlamlı olarak azaldı (disülfid için, ortalama  $\pm$  SD,  $19.6 \pm 7.4$  vs  $14.4 \pm 3.4$   $\mu\text{mol/L}$ ,  $p < 0.001$ , disülfid/natif tiyol için, medyan (IQR),  $5.8$  ( $3.8-8.1$ ) vs  $4.3$  ( $3.7-5.8$ )  $\mu\text{mol/L}$ ,  $p = 0.017$ , disülfid/total tiyol için, medyan (IQR),  $4.7$  ( $3.3-6.1$ ) vs  $3.7$  ( $3.2-4.7$ )  $\mu\text{mol/L}$ ,  $p = 0.017$ , natif/total tiyol için, medyan (IQR),  $80.9$  ( $75.3-86.5$ ) vs  $85.1$  ( $81.0-87.1$ )  $\mu\text{mol/L}$ ,  $p = 0.017$ ). ROP evreleri ile herhangi bir tiyol/disülfid parametresi arasında anlamlı fark bulunmadı (tüm değerler için  $p > 0.05$ ).

**Sonuç:** Bu bulgular ROP hastalarında tiyol/disülfid homeostazının değiştiğini göstermektedir. Bu metabolik imbalans ROP etiolojisinde önemli olabilir.

**Anahtar kelimeler:** Natif tiyol, oksidatif stres, prematüre retinopatisi, tiyol/disülfid homeostazı, total tiyol.

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## INTRODUCTION

Retinopathy of prematurity (ROP) was first termed as retrolental fibroplasia by Terry<sup>1</sup> in 1942. Although much has changed in knowledge of ROP, it is still the most prominent cause of childhood blindness worldwide.<sup>2</sup>

In order to understand ROP pathophysiology, it is important to know the signaling events that are involved and result in altered angiogenesis in ROP. There are multiple stress factors that are important in ROP, including oxidative stress (OS), altered oxygenation, nutritional complications, inflammation, and oxidative/hypoxic pathways.<sup>3</sup> It is well-documented that ROP patients are susceptible to OS in the phospholipid-rich retina, which is sensitive to lipid peroxidation<sup>4</sup> and oxygen consumption.<sup>5</sup> Oxygen fluctuations<sup>6</sup> and reactive oxygen species, produced by nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase,<sup>7</sup> can also activate vascular endothelial growth factor receptor 2 signaling and activate pathologic angiogenesis.

The imbalance develops due to OS which is a dynamic mix of disulfides, protein, and low molecular weight thiols.<sup>8</sup> Conventional measures for analyzing these disulfides/thiols were initiated in 1979; however, Erel and Neselioglu<sup>9</sup> developed a colorimetric method to measure the ratio of both thiols/disulfides simultaneously.

Thiol/disulfide homeostasis is compromised in many metabolic disorders and has a role in antioxidant protection, detoxification, signal transduction, and many other cellular functions.<sup>10,11</sup>

The goal of this study was to analyze thiol/disulfide levels in ROP patients and premature infants without ROP. To best of our knowledge, this study is the first evaluating the thiol/ disulfide balance in ROP using the colorimetric method developed by Erel and Neselioglu.<sup>9</sup>

## MATERIALS AND METHODS

We enrolled 71 premature babies that were admitted to our neonatology unit between July 2016 and June 2017. The study protocol was written in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Erciyes University in Turkey. The parents or guardians of the premature babies gave written informed consents.

The inclusion criterion was gestational age < 32 weeks or birth weight less than 1,500 grams. Infants who had major congenital anomaly, perinatal asphyxia, twin-to-twin transfusion, and intraventricular hemorrhage greater than or equal to Grade 2 or sepsis and those receiving blood transfusion or inotrope therapy and/or developed

bronchopulmonary dysplasia were excluded from the study. Moreover, premature infants having additional systemic diseases or receiving additional treatments other than routine treatment were also excluded. Only the subjects with ROP at less than or equal to stage 3 were included since during the enrollment period, the numbers of patients with ROP at stages 4 and 5 were only 3 and 2, respectively, which were very low to include in the statistical analysis.

Subjects were classified into 2 groups: patients with ROP (n = 48) and patients without ROP (n = 23). ROP was determined by an examination 4–6 weeks after birth.<sup>12</sup> Pupillary dilatation and indirect ophthalmoscopy was used to determine ROP manifestation and severity. At the first ROP screening, a single aliquot of peripheral venous blood (1 mL) was collected.

Venous blood samples were separated into serum and plasma by centrifugation; serum was stored at -80°C until analysis. Serum levels of native and total thiol as well as the ratio of disulfide to native and total thiol were analyzed by an automated colorimetric method developed by Erel and Neselioglu.<sup>9</sup> In this new method, which is used in biochemical analysis to assess serum thiol-disulfide homeostasis, the disulfide bonds (-S-S-) are reduced to functional thiols (-SH) with NaBH<sub>4</sub>, followed by NaBH<sub>4</sub> removal with formaldehyde. Ellman's reagent was used to analyze total sample thiol amounts. The dynamic disulfide content was calculated as a ratio of half of the difference between the total and native thiol. Thus, the native thiol, total thiol, and disulfide levels were measured and the percentages of disulfide-to-native, disulfide-to-total, and native-to-total thiol were calculated for all patients with and without ROP.<sup>9</sup>

## STATISTICAL ANALYSIS

The data were analyzed using the IBM SPSS Statistics version 21.0 (IBM Corp., Chicago, IL, USA). The normality of each variable was controlled with the Kolmogorov-Smirnov test. The Student's t and the Mann-Whitney U tests were used for parametric data and nonparametric data for group comparison, respectively. A one-way analysis of variance (ANOVA) test for parametric data and Kruskal-Wallis test for nonparametric data were used to compare the measures among subgroups according to stages of ROP. A p value less than 0.05 was considered statistically significant.

## RESULTS

Forty eight ROP subjects (26 boys and 22 girls) and 23 control subjects (12 boys and 11 girls) were recruited to the study. The mean weight was 1,075.0 ± 150.82 g in subjects with ROP and 1,107.82 ± 126.34 g in subjects without ROP.

The mean gestational age was  $28.85 \pm 1.36$  weeks in ROP patients and  $29.43 \pm 0.99$  weeks in controls. There were no significant differences in sex distribution, mean weight, and gestational age between the two groups ( $p = 0.875$ ,  $p = 0.051$ , and  $p = 0.062$ , respectively). Table 1 shows the demographic features in the groups.

Comparison of thiol-disulfide measurements between the ROP patients and controls are demonstrated in Table 2 and Figure 1. When we analyzed the thiol/disulfide homeostasis parameters, no significant differences were observed in serum native thiol ( $p = 0.913$ ) and total thiol between ROP patients and controls ( $p = 0.420$ ); however, native-to-total thiol ratio was significantly lower in the ROP patients ( $p = 0.017$ ). On the other hand, ROP patients had significantly higher level of disulfide as well as disulfide-to-native thiol and disulfide-to-total thiol ratios as compared with the controls ( $p < 0.001$ ,  $p = 0.017$ , and  $p = 0.017$ , respectively). The differences in all thiol/disulfide homeostasis parameters (except for native and total thiol) between the ROP patients and controls were statistically significant.

There were no significant differences among the ROP patients at stage 1 ( $n = 18$ ), stage 2 ( $n = 19$ ), and stage 3 ( $n = 11$ ) regarding the thiol/disulfide homeostasis parameters ( $p = 0.575$  for native thiol,  $p = 0.808$  for total thiol,  $p = 0.061$  for disulfide, ANOVA;  $p = 0.129$  for disulfide-to-native thiol,  $p = 0.129$  for disulfide-to-total thiol,  $p = 0.129$  for native thiol/total thiol, Kruskal-Wallis test).

## DISCUSSION

Knowledge, diagnosis, and management of ROP have been improved since the origin of high oxygen associated retrolental fibroplasia.

With improvements in neonatal care, several changes in our knowledge of ROP have occurred. New therapies to encourage physiologic retinal vascular development, vascular repair, and inhibition of vasoproliferation have been recommended.

Reducing excessive oxidative/nitrosative stress has also been studied. OS occurs when equilibrium between generation and removal of reactive oxygen species is impaired. Considerable evidence supports the view that OS has an important etiological role in ROP pathogenesis.<sup>13-17</sup>

Thiol/disulfide balance is a local and systemic OS gauge. It has a crucial role for several cellular functions, including antioxidant protection. The valuation of OS through thiol/disulfide homeostasis assay has become a topic of interest for multiple diseases. Various systemic pathologies can result from an imbalance of thiol-disulfide homeostasis, such as diabetes<sup>18</sup> and cardiovascular diseases.<sup>19</sup> Recently, many localized diseases have been associated with a deterioration in thiol-disulfide homeostasis, such as central serous chorioretinopathy,<sup>20</sup> age-related macular degeneration,<sup>21</sup> seasonal allergic rhinitis,<sup>22</sup> and acute tonsillopharyngitis.<sup>23</sup>

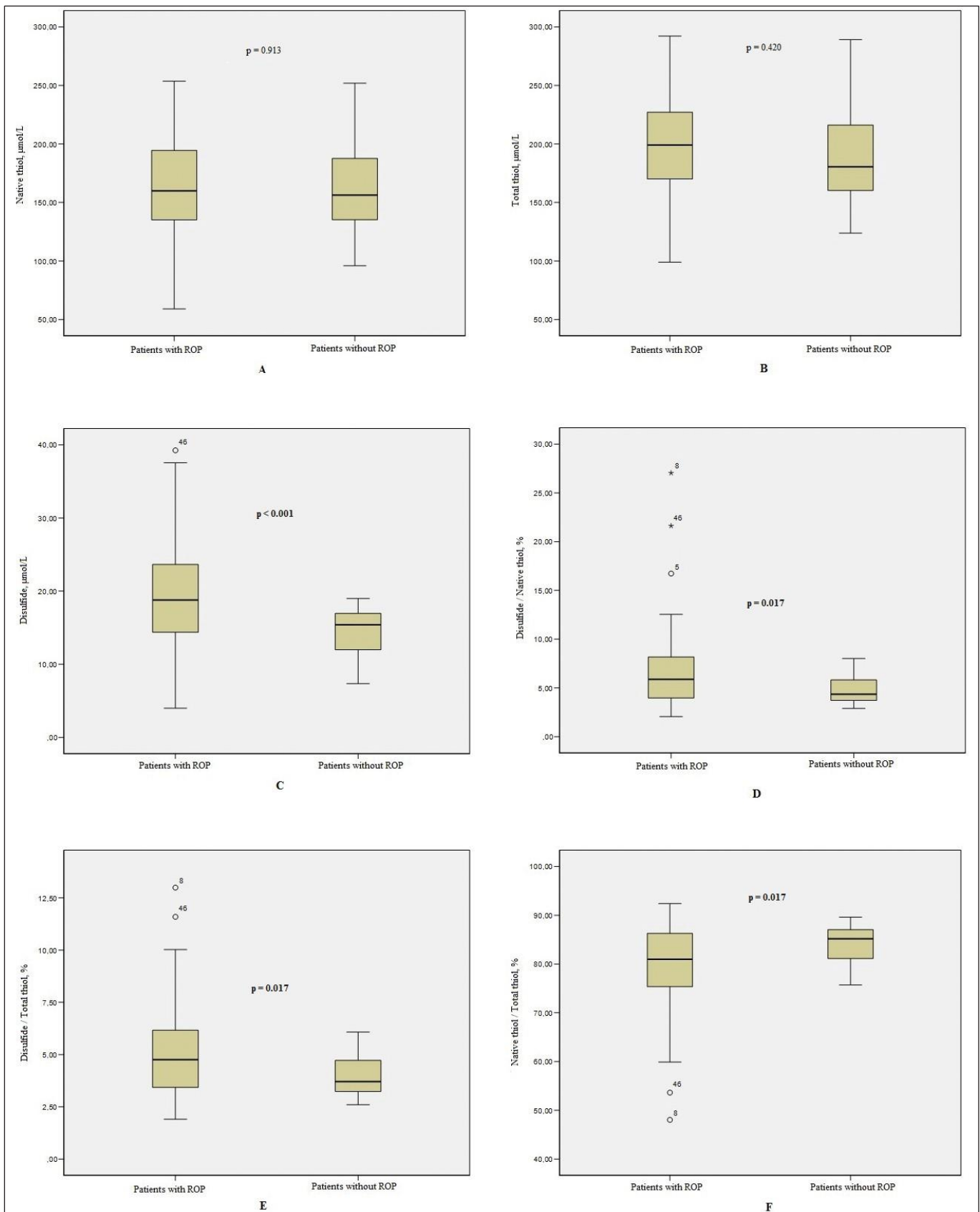
To the best of our knowledge, the relationship between thiol/disulfide homeostasis and ROP has not been

**Table 1.** Demographic features of the study groups.

Parameters	Patients with ROP (n = 48)	Patients without ROP (n = 23)	p value
Gestational age, weeks	$28.85 \pm 1.36$	$29.43 \pm 0.99$	0.062
Sex, girls / boys	22/26	11/12	0.875
Birth weight, g	$1,075.0 \pm 150.82$	$1,107.82 \pm 126.34$	0.051
ROP = Retinopathy of prematurity			

**Table 2.** Plasma thiol-disulfide levels of groups.

Parameters	Patients with ROP		Patients without ROP		p value
	Mean± SD	Median (IQR)	Mean± SD	Median (IQR)	
Native thiol, $\mu\text{mol/L}$	$159.60 \pm 44.68$	160 (134.0–195.4)	$160.82 \pm 41.02$	156.3 (132.6–191.8)	0.913
Total thiol, $\mu\text{mol/L}$	$198.93 \pm 44.65$	199.1 (170.0–227.2)	$189.76 \pm 44.56$	180.5 (159.0–225.9)	0.420
Disulfide, $\mu\text{mol/L}$	$19.66 \pm 7.44$	18.7 (14.2–23.6)	$14.46 \pm 3.42$	15.4 (11.9–17.0)	<0.001
Disulfide /Native thiol, %	$6.93 \pm 4.62$	5.8 (3.8–8.1)	$4.68 \pm 1.42$	4.3 (3.7–5.8)	0.017
Disulfide /Total thiol, %	$5.11 \pm 2.23$	4.7 (3.3–6.1)	$3.90 \pm 0.97$	3.7 (3.2–4.7)	0.017
Native thiol /Total thiol, %	$79.53 \pm 8.94$	80.9 (75.3–86.5)	$84.39 \pm 3.88$	85.1 (81.0–87.1)	0.017
ROP = Retinopathy of prematurity; IQR = Interquartile range; SD = Standard deviation					



**Figure 1.** Comparison of thiol-disulfide measurements between patients with retinopathy of prematurity and controls in terms of: (A) Native thiol; (B) Total thiol; (C) Disulfide; (D) Disulfide / Native thiol ratio; (E) Disulfide / Total thiol ratio; and (F) Native thiol / Total thiol ratio.

studied so far. In the present study, our goal was to determine thiol/disulfide homeostasis in the ROP patients in comparison with the patients without ROP using the novel colorimetric method developed by Erel and Neselioglu.<sup>9</sup> We found that native-to-total thiol ratios were lower in the blood of ROP patients. There were no significant differences for native thiol and total thiol values in the ROP patients as compared to the controls. Moreover, our study found that disulfide, disulfide-to-native thiol ratios, and disulfide-to-total thiol ratios were higher in the ROP patients than those in the controls. We suggest that decreased antioxidant status in the ROP patients has a role in this thiol/disulfide imbalance. Although there is no study in the literature that we can directly compare our findings, the findings of Ünal et al.<sup>24</sup> are valuable for this thiol/disulfide imbalance. They evaluated the dynamic thiol/disulfide homeostasis in very low birth weighted preterm infants at baseline, on week 1, and 3. Authors found that thiol levels increased in all measurements; disulfide and disulfide/thiol ratio increased on week 1 but decreased on week 3 and native-to-total thiol ratio decreased on week 1 but increased on week 3. Therefore, they attributed this shift towards disulfides in the thiol/disulfide homeostasis on week 1 to the subjection of patients to OS. In premature infants and ROP, altered oxygen, light, and metabolism have been linked to higher OS. Preterm labor and preterm birth can decrease the oxidative reserve and present higher OS in comparison to full-term.<sup>25</sup> On the other hand, Akdoğan et al.<sup>26</sup> conducted a study on 28 infants with ROP at stage 2 and higher to determine the effects of a potent antioxidant on thiol/disulfide homeostasis. However, they found no differences in the total thiol, native thiol, and disulphide levels as well as native/total thiol ratio and disulphide/total ratio after two weeks of antioxidant administration.

Mounting evidence has shown that NADPH oxidase is important in experimental oxygen-induced retinopathy models. This method works to generate reactive oxygen species that restrict peripheral retina vascularization and mediation of later vasoproliferation into the vitreous.<sup>27</sup>

Hyperoxia-induced apoptosis occurs via nitrosative stress from peroxynitrite.<sup>28-30</sup> The formation of peroxynitrite from nitric oxide occurs in the presence of increased superoxide radicals. Arginase-2 has been demonstrated to have role in peroxynitrite-mediated neuro-glial injury<sup>31</sup> and vaso-obliteration in oxygen-induced retinopathy.<sup>32</sup>

In previous report, Ateş et al. found higher mean plasma and urine 8-hydroxy 2-deoxyguanosine and malondialdehyde levels in patients with ROP compared to controls, suggesting that 8-hydroxy 2-deoxyguanosine in leukocyte DNA and urine may be a useful biomarker for the evaluation of oxidative damage in patients with ROP.<sup>33</sup>

The use of anti-oxidants, including superoxide dismutase in liposomes<sup>34</sup> or apocynin,<sup>35</sup> reduced avascular retina in phase 1 of the rat oxygen-induced retinopathy model but these substances did not reduce intravitreal neovascularization in phase 2 in the rat oxygen-induced retinopathy model.

Antioxidants may be effective for blocking reactive oxygen species, but may not have the ability to affect those that trigger pathologic intracellular signaling molecules. Studies have expanded our knowledge on the oxidative and nitrosative compounds in ROP and show potential as future treatment options. Oxidative/nitrosative balance is necessary and more studies are warranted on this issue.<sup>16</sup>

The main limitation of our study is its systemic evaluation of thiol/disulfide homeostasis. Our results do not indicate that the OS increased in vitreous. Ideally, thiol disulfide levels should be measured from vitreous. However, using this technique is not presently possible, since most thiol molecules are located in albumin. The next limitation of the study is the lack of comparison between these parameters and the patients in severe stages of (stage 4 and stage 5) ROP and controls. More longitudinal analyses are required for precise delineation of these implications in severe ROP patients.

In conclusion, our results showed an impaired systemic thiol/disulfide homeostasis in the ROP subjects compared to premature infants without ROP. This imbalance suggests the role of decreased systemic antioxidant status in ROP development.

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